Study of biological effects and oxidative stress related responses in gamma irradiated *Arabidopsis thaliana* plants

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Abstract. This study aimed at investigating biological effects in *Arabidopsis thaliana* leaves and roots irradiated for 72 h with 3.5 Gy or 30 Gy of gamma radiation, and to unravel oxidative stress related responses to achieve a better understanding of the importance of the cellular redox balance as a modulator in gamma radiation stress. *A. thaliana* performs like a rather radioresistant plant species as no alterations on growth and only minor alterations in the nutrient profile were observed. Gamma irradiation did not seem to induce an NADPH mediated oxidative burst and lipid peroxidation appeared to be directly induced by ionizing radiation rather than mediated through LOX activity. As ionizing radiation can cause indirect damage via water radiolysis, H_2O_2 is hypothesized to be an important reactive oxygen species under radiation stress. Although most H_2O_2-scavenging enzymes remained unchanged, important alterations were observed for *CAT1*, *CAT2* and *CAT3* expression.

1. INTRODUCTION

Besides various natural sources of ionizing radiation in the environment, anthropogenic activities such as nuclear power production, nuclear accidents and weapon testing can cause an enhancement of the environmental radiation dose. On international level there is imminent legislation for the environmental protection against ionizing radiation. Therefore it is important to study the effects and unravel the mechanisms by which organisms, including plants, respond to gamma radiation.

While exposure to high radiation doses causes death of organisms, previous studies on plants have indicated that exposure to lower ionizing radiation doses can cause growth reduction, a decreased reproduction capacity, DNA damage and morphological changes [1-4]. Besides various genetic and morbidity endpoints, irradiation with relatively low doses can also cause a stimulation of growth as reviewed by Sax [5]. This review has also indicated that the radiosensitivity of plants is dependent on the species and growth stage in which

they are irradiated [5]. It has also been assumed that the radiosensitivity is proportional with the size of the genome indicating that plants with a very small genome are more radioresistant than plants with a larger genome [6]. Pine trees are known to be very radiosensitive and while irradiation with 0.1-1 Gy already caused low injuries, irradiation with more than 60 Gy resulted in a massive mortality and no regeneration of pine trees after the Chernobyl accident [7]. For *A. thaliana* seedlings on the other hand, Wi et al. [8] demonstrated that seedlings developed normally after exposure to 5 Gy, while plant height was significantly reduced following exposure to 50 Gy. In general, a screening value, representing a dose rate affecting 5 % of the plant species at a 10 % level, presented as a predicted no effect dose rate, of 70 µGy h^{-1} was derived for plants [9].

Ionizing radiation can react directly with macromolecules causing immediate cellular damage such as DNA strand breaks, lipid oxidation and protein inactivation. As water is an important constituent of all living cells, cellular damage can also be initiated by reactive oxygen species (ROS) that are generated during the radiolysis of water, an important pathway under gamma irradiation [10]. Plant cells have a well-equipped antioxidative defense system comprising enzymes and metabolites to regulate the amount of ROS [11]. Although the induction of the antioxidative defense system seems an important stress response mechanism after gamma irradiation [12-15], information remains limited.

This study aimed at investigating biological effects in *A. thaliana* leaves and roots after irradiation with 3.5 Gy or 30 Gy and to unravel oxidative stress related responses to achieve a better understanding of the importance of the cellular redox balance as a modulator in gamma radiation stress. Several endpoints such as growth, nutrient profile, lipid peroxidation, antioxidative enzyme capacities and gene expression of ROS-producing and -scavenging enzymes were analyzed.

2. MATERIALS AND METHODS

2.1 Plant culture and gamma irradiation

A. thaliana seeds (Columbia ecotype) were spread on moist filter paper at 4 °C for 3 days to synchronize germination. Subsequently, the seeds were sown on plugs from 1.5 ml polyethylene centrifuge tubes filled with 2 % agar (Difco). The plugs were positioned in a PVC cover capable of holding 36 plugs. Next, the cover was placed on a container filled with 1.35 l of a modified Hoagland solution. Plants were grown in a growth chamber under a 14 h photoperiod (photosynthetic photon flux density of 200 µmol m^{-2} s^{-1} at the leaf level), with day/night temperatures of 22 °C/18 °C and 65 % relative humidity. Subsequently, 18-day-old plants were transferred to the radiation unit of SCK•CEN where they were irradiated with gamma radiation from a ^{137}Cs source (1.25×10^{12} Bq) during 72 h. Plants received a total dose of 3.5 Gy with an average dose rate of 50 mGy h^{-1} or a total dose of 30 Gy with an average dose rate of 400 mGy h^{-1}. Leaves and roots were harvested as ± 100 mg samples, snap frozen in liquid nitrogen and stored at -80 °C. Fresh weight was determined for at least 20 biological replicates. Samples for nutrient analyses were dried for 1 week at 70 °C. Leaves were rinsed with distilled water and roots were washed twice for 10 min with 1 mM $Pb(NO_3)_2$ at 4 °C and once for 10 min with distilled water.

2.2 Nutrient concentrations

After dry-ashing using a muffle furnace, dried plant material was digested in 0.1 M HCl for determination of several nutrients. Copper, iron, manganese and zinc concentrations were determined using inductively coupled plasma mass spectrometry (Perkin-Elmer). Concentrations of calcium, potassium and magnesium were determined using suppressed ion chromatography (Dionex).

2.3 Lipid peroxidation

Thiobarbituric acid (TBA) reactive compounds were used as measure of lipid peroxidation in *A. thaliana* leaves. Leaf tissue (± 100 mg) was homogenized with 2 ml 0.1 % TCA (trichloroacetic acid) buffer using a mortar and pestle. After centrifugation for 10 min at 20 000 × g, 0.5 ml of the supernatant was added to 2 ml 0.5 % TBA. This mixture was heated for 30 min at 95 °C and quickly cooled in an ice bath. After centrifugation for 10 min at 20 000 × g, the absorbance of the supernatant was measured spectrophotometrically at 532 nm corrected for unspecific absorbance at 600 nm [16].

2.4 Enzyme capacities

Frozen leaf or root tissue (± 100 mg) was homogenized in ice cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 4 % insoluble polyvinylpyrrolidone (2 ml buffer 100 mg^{-1} FW) using a mortar and pestle. The homogenate was squeezed through a nylon mesh and centrifuged at 20 000 × g and 4 °C for 10 min. The enzyme capacities were measured spectrophotometrically in the supernatant at 25 °C. Guaiacol peroxidase (GPX) and syringaldazine peroxidase (SPX) capacities were measured at 436 nm and 530 nm according to Bergmeyer et al. [17] and Imberty et al. [18] respectively. Ascorbate peroxidase (APX) capacity was measured at 298 nm following the method of Gerbling et al. [19]. Analysis of superoxide dismutase (SOD) capacity was based on the inhibition of cytochrome c at 550 nm according to McCord and Fridovich [20]. Analysis of the catalase (CAT) capacity was performed as described by Bergmeyer et al. [17].

2.5 Gene expression

Frozen leaf or root tissue (± 100 mg) was ground thoroughly in liquid nitrogen using a mortar and pestle. After RNA extraction (RNeasy Plant Mini Kit, Qiagen), followed by the determination of the RNA quantity (Nanodrop, Isogen Life Science) and quality (Bioanalyzer, Agilent Technologies), first strand cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen) and equal amounts of starting material were used (1 µg). Quantitative real time PCR was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems), using Sybr Green chemistry. Gene expression data were normalized against multiple housekeeping genes according to Remans et al. [21] and presented relative to the control treatment.

2.6 Statistical analyses

Statistical analyses were performed using an ANOVA test in SAS 9.1. The ANOVA test was performed separately for leaves and roots. Mean values for the different treatments were compared using Tukey's multiple comparison tests.

Transformations were applied when necessary to approximate the assumptions of normality and same error variance.

3. RESULTS AND DISCUSSION

Gamma irradiation of 18-day-old *A. thaliana* seedlings for 72 h with a total dose of 3.5 Gy or 30 Gy did not cause any difference in fresh weight for leaves or roots (results not shown). In contrast to these results, we expected to see a reduction in growth because the lowest total dose of 3.5 Gy was chosen based on a previous experiment inducing a 30 % plant growth reduction after 7 weeks exposure to the same total dose [22]. The reason why we did not see a reduction in growth lies probably in the difference between acute and chronic exposure. A previous study by Kovalchuk et al. [4] already indicated with transcriptome analysis that there are fundamental differences in plant responses between acute and chronic exposure to ionizing radiation. Furthermore, based on the differences in screening values for chronic (10 µGy h^{-1} for all ecosystems) versus acute exposure (300 mGy for terrestrial ecosystems), it can be shown that a much higher dose can be applied acute without seeing effects while a lower dose already induces effects when applied chronically [23]. Based on these findings it can be concluded that although the total dose applied induced effects in a chronic exposure setup, these effects could not be extrapolated to our more acute setup. Likewise, observations from acute exposure situations cannot be extrapolated to chronic exposure situations. Previous studies have also reported divergent results depending on the sensitivity of the plant species and the radiation dose applied. Wi et al. [8] reported no alterations in the height of *A. thaliana* seedlings 12 days after gamma irradiation up to 5 Gy, while exposure to 50 Gy significantly decreased plant height. Irradiation effects on two generations of *P. sativum* seedlings were studied by Zaka et al. [3] reporting doses above 6 Gy significantly inhibited plant growth and productivity in the first generation while the effects were even more severe in the second generation. For red pepper plants on the other hand, Kim et al. [14] reported growth stimulating effects after exposure to relatively low doses of 2 and 4 Gy, while exposure to 8 and 16 Gy inhibited growth again. As the radiosensitivity is suggested to be proportional to the size of the genome, *A. thaliana*, due to its relatively small genome, can probably be classified as a rather radioresistant plant species as was also suggested by Daly and Thompson [1].

For the nutrient profile, leaves of irradiated *A. thaliana* seedlings seem more affected than roots. Exposure to the highest radiation dose of 30 Gy caused a significant decrease in calcium, magnesium, copper and manganese concentrations in the leaves (table 1). The manganese concentration in the leaves was already significantly decreased after exposure to the lower radiation dose of 3.5 Gy while this concentration was significantly enhanced in roots irradiated with the high radiation dose (table 1). These decreases in nutrient concentrations were, although significant, only minor and had no effect on growth and development during this short exposure period.

Table 1 - Nutrient concentrations in leaves and roots of irradiated *Arabidopsis thaliana*

		LEAVES			ROOTS		
Nutrient		**Control**	**3.5 Gy**	**30 Gy**	**Control**	**3.5 Gy**	**30 Gy**
[mg g^{-1}]	*Ca*	41.6±1.5^{a}	40.2±0.3^{ab}	36.3±1.2^{b}	7.0±1.5^{A}	5.1±0.2^{A}	6.2±1.4^{A}
	K	30.7±2.0^{a}	33.1±0.4^{a}	30.1±0.9^{a}	12.9±4.6^{A}	15.4±1.8^{A}	16.3±7.4^{A}
	Mg	9.1±0.3^{a}	8.8±0.1^{ab}	8.2±0.2^{b}	2.2±0.2^{A}	1.8±0.1^{A}	1.9±0.2^{A}
[µg g^{-1}]	*Cu*	10.0±1.0^{a}	7.7±0.8^{ab}	6.7±0.1^{b}	14.5±3.6^{A}	15.9±3.5^{A}	11.9±3.6^{A}

g g^{-1} DW]							
	Fe	-	-	-	1127.4±54.4^{A}	997.0±30.6$_{A}$	969.6±48.1$_{A}$
	Mn	135.2±3.0^{a}	118.0±2.7^{b}	122.6±1.3^{b}	217.2±8.4^{A}	223.6±24.0$_{A}$	311.2±22.0$_{B}$
	Zn	31.1±1.7^{a}	28.5±1.2^{a}	27.4±0.2^{a}	77.4±4.4^{A}	80.9±1.5^{A}	89.9±10.2^{A}

Data represent the mean ± S.E. of at least 5 biological replicates. Different letters indicate significant differences between the treatments for leaves (small letters) and roots (capital letters) ($p < 0.05$).

One of the earliest responses of plant cells under biotic and abiotic stress situations is the oxidative burst during which enhanced levels of ROS are produced. ROS have therefore a dual role as both toxic byproducts of aerobic metabolism and key regulators of biological processes such as growth, cell cycle and response mechanisms to various stress situations [11, 24]. As plasma membrane related superoxide producing NADPH oxidases can be a possible source for ROS during this oxidative burst, gene expression levels for several NADPH oxidase isoforms were investigated for irradiated *A. thaliana* leaves and roots but no alterations were observed (results not shown). These results suggest that ROS production under ionizing radiation stress is probably only indirectly due to water radiolysis and not via an oxidative burst at the plasma membrane level.

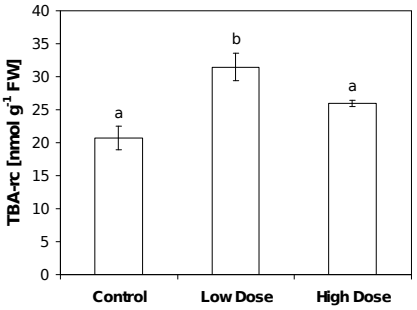

Figure 1 - The level of lipid peroxidation, based on the amount of TBA-reactive compounds [nmol g^{-1} FW], in leaves exposed to a low dose (3.5 Gy), a high dose (30 Gy) and the control. Each point represents the mean ± S.E. of 5 biological replicates. Different letters indicate significant differences ($p < 0.05$).

Lipid peroxidation can be initiated via immediate interaction with ionizing radiation, but also indirectly by interaction with various ROS, produced under stress conditions. The amount of TBA reactive compounds in the leaves was determined as a measure for the level of lipid peroxidation. An unusual result was observed: exposure to the low radiation dose caused a significant increase in TBA reactive compounds affecting membrane structure and function, while no alterations were observed following exposure to the highest radiation dose (figure 1). For *P. sativum* plants exposed to UV-B radiation, Agrawal and Mishra [25] also reported a significant increase in the level of lipid peroxidation. Besides being initiated directly or by a number of ROS, lipid peroxidation in plant cells can also be initiated by the enzyme lipoxygenase (LOX) [26]. Transcriptional levels of two LOX isoforms *LOX1* (results not shown) and *LOX2* (figure 2D) were investigated but no alterations in gene expression were observed indicating lipid peroxidation is probably not induced via this enzymatic pathway.

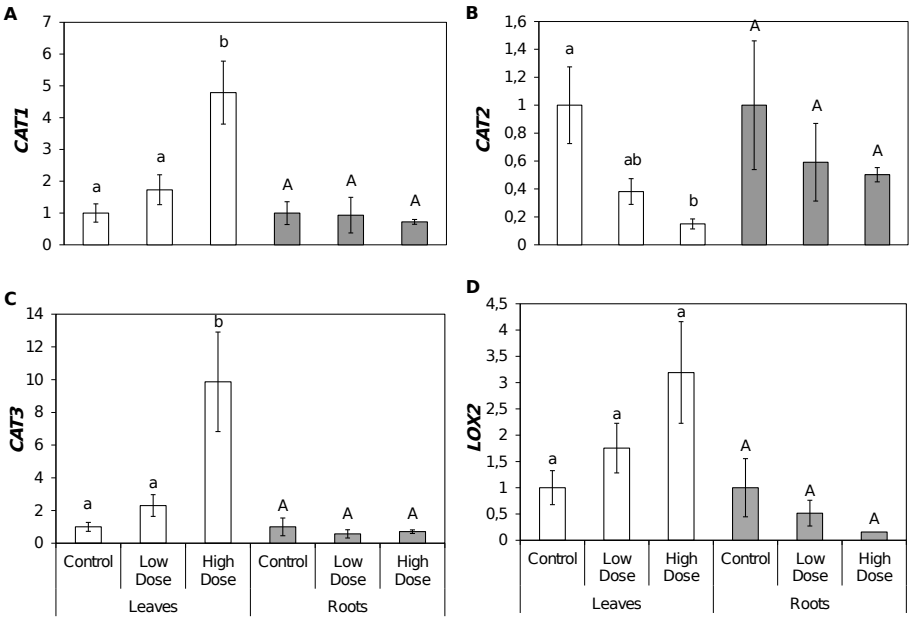

Figure 2 - Gene expression of H_2O_2-scavenging enzymes (A-C) and a ROS-producing enzyme (D) in leaves (white bars) and roots (grey bars) of *A. thaliana* exposed to a low dose (3.5 Gy), a high dose (30 Gy) and the control. Transcript levels are expressed relatively to the control for leaves and roots separately and data points represent the mean ± S.E. of 4 biological replicates. Different letters above the bars indicate significant differences between the treatments for leaves (small letters) and roots (capital letters) ($p < 0.05$).

Table 2 - Capacities of antioxidative enzymes [U g^{-1} FW] in leaves and roots of irradiated *A. thaliana*

	LEAVES			ROOTS		
Enzyme	**Control**	**3.5 Gy**	**30 Gy**	**Control**	**3.5 Gy**	**30 Gy**
APX	9.63±1.54^{a}	12.78±2.59^{a}	13.24±1.88$_{a}$	2.83±1.03^{A}	3.61±1.25^{A}	4.30±1.26^{A}
CAT	0.55±0.10^{a}	0.75±0.03^{a}	0.72±0.04^{a}	0.14±0.02^{A}	0.14±0.01^{A}	0.14±0.01^{A}
GPX	1.08±0.16^{a}	1.50±0.20^{a}	1.39±0.09^{a}	16.61±1.60^{A}	18.60±0.70^{A}	17.99±1.29$_{A}$
SOD	61.33±9.02^{a}	78.60±6.60^{a}	64.43±11.14^{a}	64.45±8.36^{A}	56.20±4.90^{A}	61.81±13.42^{A}
SPX	1.78±0.45^{a}	2.40±0.40^{a}	2.64±0.50^{a}	9.77±1.38^{A}	11.30±1.40^{A}	12.29±1.34$_{A}$

Data represent the mean ± S.E. of at least 5 biological replicates. Different letters indicate significant differences between the treatments for leaves (small letters) and roots (capital letters) ($p < 0.05$).

Plant cells possess a well-equipped antioxidative defense system to detoxify and regulate ROS under both normal metabolism and stress situations. Superoxide dismutase constitutes the first line of defense against ROS. To investigate the role and importance of SOD in response to ionizing radiation, its capacity (table 2) was investigated together with gene expression analyses of several isoforms (*CSD1-3, FSD1-3, MSD1*), but no alterations were observed (results not shown). This could possibly indicate that no or only little $O_2^{\bullet-}$ is directly produced via energy transfer from the radiation field to molecular oxygen. As *A. thaliana* is probably a rather radioresistant plant, it could be possible that the radiation dose applied was insufficient to induce SOD

alterations as Kim et al. [14] reported an increase in SOD capacities after irradiation of red pepper plants with doses ranging from 2 to 16 Gy.

Several enzymes such as CAT and PX regulate the detoxification of H_2O_2 into water in plant cells. Although no alterations in CAT capacity (table 2) were observed for irradiated leaves and roots, important variations were observed on transcriptional level for 3 CAT isoforms in irradiated leaves. While *CAT1* and *CAT3* transcript factors were significantly up-regulated after exposure to the highest radiation dose (figure 2A, C), *CAT2* expression was significantly down regulated after exposure to the highest radiation dose (figure 2B). As H_2O_2 is an important water radiolysis product, the enhanced CAT expression, although not yet visible on protein level, indicates an enhanced defense against ionizing radiation induced ROS. The up- and down-regulations of the various CAT isoforms could compensate for each other explaining the unaffected protein capacity.

4. CONCLUSIONS

A. thaliana seems to be a rather radioresistant plant species as no alterations on growth and only minor alterations on the nutrient profile and the antioxidative defense system were observed after irradiation with a total gamma radiation dose of 3.5 or 30 Gy applied over a 3 days period. Although an early oxidative burst is an important response mechanism under other stress situations, irradiation with gamma radiation appeared not to induce an NADPH mediated ROS production. Lipid peroxidation seemed to be directly induced by ionizing radiation and not mediated through LOX activity. As ionizing radiation can also cause indirect damage via water radiolysis, H_2O_2 is hypothesized an important ROS present under ionizing radiation stress. Although most H_2O_2 scavenging enzymes remained unaltered, an important role for CAT was indicated as important alterations were observed on transcriptional level for 3 CAT isoforms.

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